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The occurrence of clinical signs of toxicity and observable effects on and around the injection site following iv injection for 7 days of a single, daily dose of an artelnic acid/lysine (AL/lysine) formulation, equal to or greater than the anticipated clinical dose, was investigated in Beagle dogs. Groups consisting of one or two male and one or two female dogs were given an iv dose of 2 or 4 mL of an AL/lysine formulation containing 0, 10, or 30 mg/mL of artelnic acid daily for 7 days. All doses were delivered into a peripheral leg vein over an approximate 10-minute interval, using an infusion pump. On Days 1-14, each dog was observed at least twice daily for signs of clinical toxicity and the site of injection was closely examined for irritation/swelling or other adverse reactions. No clinical signs of toxicity or adverse reactions at the injection site were observed for any dogs given the vehicle control formulation (either 2 mL or 4 mL), or for dogs given 2 mL of either the 10 or 30 mg/mL AL/lysine formulation, or for the male dogs given 4 mL of the 30 mg/mL AL/lysine formulation. The two female dogs given 4 mL of 30 mg/mL AL/lysine displayed transient swelling in the leg, near the injection site, beginning on Day 5 or 6 of dosing and continuing through Day 10 or 11; no necrosis, discoloration, or other adverse signs were evident in these two animals. The results of this study indicated that an injectable formulation of artelnic acid/lysine was well tolerated by dogs given a single iv dose for 7 consecutive days. In future work, the pharmacodynamic effects of iv administered artelnic acid and artesunic acid, as assessed by signs of clinical and pathological toxicity, will be investigated in rats.

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1.0 INTRODUCTION

The work scope of this contract involves the performance of studies in rats and dogs on the pharmacokinetic and pharmacodynamic properties of drugs under clinical development by the U.S. Army Medical Research and Development Command. The pharmacokinetic aspect of these studies involves an investigation of the absorption, disposition, metabolism (biotransformation), and elimination of test compounds in experimental animals. The pharmacodynamic aspect involves relating certain measured parameters, for example, the production of methemoglobin, to blood and plasma levels of the test compound and/or its metabolites, or assessing toxicological parameters such as clinical signs and mortality occurring after administration of a test compound. The information derived from these studies is intended to provide a data base for establishing an appropriate species and appropriate doses for subsequent subchronic and chronic toxicity studies, to predict for possible organ toxicities which might occur, and to generate data required by the Food and Drug Administration prior to submission of a Notice of Claimed Investigational Exemption for a New Drug (IND) and New Drug Applications, Human Use (NDA).

During the past year of the contract, a pharmacodynamic/toxicological study was conducted in dogs with the anti-malarial agent, artelinic acid. The purpose of this study was to assess the occurrence of clinical signs of toxicity and observable effects on and around the injection site following intravenous injection for 7 days of a single, daily dose of an artelinic acid/lysine formulation, equal to or greater than the anticipated clinical dose, to dogs. In addition, a task order was initiated to investigate the pharmacodynamic effects of artelinic acid and artesunic acid, as assessed by signs of clinical and pathological toxicity, following daily intravenous administration to rats for 7 consecutive days.

2.0 RESEARCH ACCOMPLISHMENTS FOR EACH TASK ORDER

2.1 Task Order SR00-1: Study of Injectable Artelinic Acid in Dogs

2.1.1 Background and Objectives

Plasmodium falciparum malaria affects 300-500 million people annually, resulting in 1-1.3 million deaths a year (WHO 2000). The high mortality is attributable to severe anemia and organ dysfunction from sequestration of infected erythrocytes to post-capillary venules, causing vessel occlusion and ischemia.

Mefloquine, halofantrine, and doxycycline are products of the U.S. Army's drug development program aimed at treating malaria. The current main effort is to develop a parenteral drug for severe malaria (to replace quinidine, the only parenteral antimalarial available in the US). Artemisinin derivatives differ from quinine/quinidine, the traditional treatments of severe malaria, in that they kill parasites much more rapidly. In that most mortality from severe malaria occurs in the first 24-48 hours, this class of compounds should theoretically reduce the mortality rate. The results of several large well-controlled studies, however, do not support this. It has been proposed that the lack of improvement may be due to slow and/or poor absorption following intramuscular administration of these oil soluble derivatives. Artesunate suppositories are being developed for initiation of treatment of critically ill patients where medical facilities are not available; however, bioavailability and variability of absorption are also an issue with suppositories.

Previously, we conducted initial range finding evaluations on the dose-related pharmacodynamic effects of artelinic acid (AL), as assessed by signs of clinical and pathological toxicity, in rats and in dogs following administration by oral gavage for 14 consecutive days. In these studies, AL was administered as a suspension in 1% carboxymethylcellulose:0.2% Tween 80. Subsequent to the completion of the originally planned definitive studies in rats and dogs with orally administered AL, the focus was changed to pursue development of an intravenous (iv) formulation of AL; thus, all work scheduled to be conducted with oral formulations of AL was terminated.

A major advantage of iv dosing is that it ensures 100% bioavailability with immediate peak concentrations. IV artesunate (AS) is currently available in China; however, only AL appears to have sufficient stability in solution to allow the drug to be available in a "ready-to-use" injection vial. The currently marketed AS formulation is a freeze-dried preparation, which requires mixing with a sodium bicarbonate solution at the bedside and then dilution with dextrose in water (Package Insert, Artesunate for Injection, Atlantic Pharmaceutical Co., Bangkok).

An AL/lysine formulation has recently been developed and is being evaluated for suitability as an iv injectable solution. In initial studies, it was found that when AL in lysine at a concentration of 32 mg/mL was administered to rats via a tail vein at a dose of 16 mg/kg daily for 3 days, venulitis, as evidenced by edema and color change, was seen. It was hypothesized that the reason for AL venotoxicity was the small size of the vein used for AL injection. If so, AL

should not be toxic to dog peripheral vein and would, therefore, be appropriate for iv treatment of humans. The clinical dose of artesunate, and perhaps artelinate, is 2 mg/kg over 5-10 minutes using a 10 mg/mL solution.

The objective of this task order was to assess the occurrence of any clinical and/or observable effects on and around the injection site following iv injection of single, daily doses of an AL/lysine formulation, equal to or greater than the anticipated clinical doses, to dogs for 7 consecutive days.

2.1.2 Materials and Methods

2.1.2.1. Test System. The male and female dogs used in this study were obtained from Marshall Farms (North Rose, NY). On the day of dosing, the dogs were 8-9 months of age and weighed between 7 and 9 kg.

Upon arrival, each dog was placed in quarantine and given a physical examination. There were no significant findings indicative of poor health, and the laboratory veterinarian released these animals for study. Housing, feed, and water procedures remained the same during the quarantine and study periods.

The dogs were exposed to their daily ration of commercial, dry Certified Canine Diet #5007 (PMI Feeds, Inc., St. Louis, MO) for a total period of approximately 2 hours per day. The quantity of the daily ration was sufficient to meet nutritional requirements. The water source was the public supply (Birmingham public water supply) and was given *ad libitum*. The dogs were individually housed in stainless steel cages in a room that was maintained at a temperature of 69-75°F and a relative humidity of 35-65%. Room lights were controlled by an automatic timer set to provide 12 hours of light (0600 to 1800 hours, CST) and 12 hours of dark per day. Cage size and animal care conformed to the guidelines of the *Guide for the Care and Use of Laboratory Animals*, 7th edition⁽¹⁾ and the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198) and to the applicable Standard Operating Procedures (SOPs) of Southern Research.

Each dog was identified by an ear tattoo or letter combination. Dogs are an accepted species to support pharmacological and toxicological evaluations of drugs used or intended for use in humans.

2.1.2.2 Test Articles: One shipment containing 30 grams of artelinic acid/lysine salt (AL/lysine) was supplied by Walter Reed Army Institute of Research (WRAIR), Washington, DC. The test article was stored frozen until used and is considered stable as a powder when stored under these conditions.

2.1.2.3 Control Articles: The control articles used for the preparation of AL/lysine dose formulations are listed below.

- Purified L-lysine (supplied by WRAIR; expiration date not supplied; stored frozen at approximately -20°C)

- L-lysine (Aldrich Chemical Company; Milwaukee, WI; Lot 08325JI; Southern Research assigned expiration date January 31, 2003; stored at room temperature)
- Sterile water (Phoenix Pharmaceutical, Inc.; St. Joseph, MO; Lot 101002F; expiration date January 2004; stored at room temperature)
- Sterile saline (Phoenix Pharmaceutical, Inc.; St. Joseph, MO; Lot 0081050; expiration date August 2003; stored at room temperature)
- Hydrochloric acid (HCl; EM Sciences; Gibbstown, NJ; Lot 32232; Southern Research assigned expiration date January 31, 2003; stored at room temperature)

2.1.2.4 Dose Formulation Preparation: The vehicle control formulations were prepared by dissolving L-lysine in sterile saline to yield a final concentration of 11.5 mg/mL of L-lysine. For preparation, the L-lysine was weighed into a volumetric flask and diluted to the desired volume with sterile saline. The contents were stirred until well mixed and then the solution was adjusted to pH 9 with hydrochloric acid. The vehicle control formulations were then filter sterilized using a 0.2 µm cellulose acetate filter and used for dosing within 7 days after preparation.

AL/lysine dose formulations were prepared by making a stock solution containing 30 mg/mL of AL; the artelinic acid content of the preformulated AL/lysine salt was equivalent to 1 gram of artelinic acid per 1.35 gram of salt. The required amount of AL/lysine salt was weighed into a volumetric flask and QS to the mark with a solution containing 0.45% saline/0.1% purified L-lysine. The contents were stirred until well mixed and then filter sterilized using a 0.2 µm cellulose acetate filter. Next, a 10 mg/mL artelinic acid formulation was made by a 1 to 3 dilution of the 30 mg/mL stock formulation. This was prepared by diluting the required amount of 30 mg/mL formulation with a solution containing 0.9% saline/0.1% purified L-lysine and filter sterilizing using a 0.2 µm cellulose acetate filter.

Due to the fact that an insufficient quantity of purified L-lysine was received from WRAIR, a 1.15% solution of L-lysine, prepared from material purchased from Aldrich Chemical Company (97% purity), was used for some of the doses; specifically, this solution was administered to the dogs in the vehicle control group (Groups 1 and 2) on Days 5-7. For all other doses and for preparation of the artelinic acid formulations, the purified L-lysine was used.

All dose formulations were stored refrigerated in amber glass vials prior to use. Each formulation was used for dosing within 7 days after preparation and was considered stable for the period of use.

2.1.2.5 Dose Formulation Analyses: Each AL/lysine dose formulation was analyzed by HPLC at the time of preparation and at the end of the dosing period for chemical concentration.

2.1.3 Experimental Design

2.1.3.1 Group Assignment: The male and female dogs were randomly assigned to five dose groups using a computer generated Artemis randomization procedure. The dogs were weighed during Week -1, and these body weights were used to randomly assign the animals to one of 4 treatment groups or to a vehicle control group (1 dog/sex/dose for Groups 1 and 2; or 2 dogs/sex for Groups 3-5) as shown in the table below:

Group ID	Formulation	Approx. Dose ^a (mg/kg/day)	Dose Conc. (mg/mL)	Dose Volume (mL/day)	No. of Males	No. of Females
1	Vehicle Control	0	0	2	1	1
2	Vehicle Control	0	0	4	1	1
3	Artelinic Acid/Lysine	2	10	2	2	2
4	Artelinic Acid/Lysine	6	30	2	2	2
5	Artelinic Acid/Lysine	12	30	4	2	2

^a Doses are based on a body weight of 10 kg.

2.1.3.2 Dose Procedure: Beginning on Day 1, each dog was given an iv dose of either the test or control article. The dose volumes were as indicated in the above table. All doses were administered into a peripheral leg vein and delivered over an approximate 10-minute interval, using an infusion pump. For each dog, the dose was administered in the same vein each day, where possible.

2.1.3.3. Body Weights: Body weights for all animals were obtained during Week -1 and on Days 1-7 (prior to dosing).

2.1.3.4. Clinical Observations: On Days 1-14, each dog was observed at least twice daily for signs of clinical toxicity. Each evaluation included an assessment of posture, activity, level of arousal, and breathing. The site of injection was closely examined for irritation/swelling or other adverse reactions.

2.1.4 Results

2.1.4.1 Clinical Observations: No adverse reactions at the injection site or clinical signs of toxicity were observed for any dogs given the vehicle control formulation (either 2 mL or 4 mL; Groups 1 and 2), or for dogs given 2 mL of either the 10 or 30 mg/mL AL/lysine formulation (Groups 3 and 4), or for the male dogs given 4 mL of the 30 mg/mL AL/lysine formulation (Group 5). The only adverse reactions noted were for the two female dogs given 4 mL of 30 mg/mL AL/lysine (Group 5). These two dogs displayed swelling in the leg, near the injection site, beginning on Day 5 or 6 of dosing and continuing through Day 10 or 11; no necrosis, discoloration, or other adverse signs were evident. Note: For one of these female dogs in Group 5, difficulty was encountered inserting the catheter for infusion into the front leg vein on each day of dosing; for this reason, not all doses given to this dog were delivered into the same leg vein.

2.1.4.2 Dose Formulations: At the time of preparation the dose formulations of AL/lysine and L-lysine were clear and colorless. The experimentally determined concentration of AL in the two AL/lysine dose formulations prior to dosing was 10.1 and 30.8 mg/mL. Thus, the concentration of AL in each formulation was within 3% of the theoretical concentration.

The dose formulations were carefully evaluated after the end of the 7-day dosing period. This evaluation included a visual inspection for color/precipitation, pH determination, and HPLC analysis for AL concentration.

Upon visual examination 8 days after preparation, the vehicle control formulation and the 10 and 30 mg/mL AL/lysine formulations appeared clear; no particulate matter was evident in any of these three formulations. The vehicle control formulation was colorless; however, the two formulations of AL/lysine were pink in appearance. The pink color was darker in the 30 mg/mL formulation than in the 10 mg/mL formulation. The pinkish appearance of the AL/lysine formulations was not evident when the formulations were drawn into a 5 cc syringe, as was used for dose administration. Due to the fact that the dose formulations were maintained in amber colored bottles, it was not known when the color change occurred. At the end of the dosing period, the pH of the 10 mg/mL AL/lysine formulation was 8.4 and that of the 30 mg/mL formulation was 8.2. [Note: pH determinations were not made on the formulations at the time of preparation].

The results of the post-dose HPLC analyses indicated the concentration of AL in the 10 and 30 mg/mL AL/lysine formulations did not change during the period of use. After the end of the dosing period, the AL content of the two formulations was determined to be 10.0 and 30.1 mg/mL.

2.1.5 Discussion

The results of this study indicated that an iv administered formulation of AL/lysine was well tolerated by dogs given a single iv dose, equal to or greater than the anticipated clinical dose, for 7 consecutive days. No discernible clinical signs of toxicity were noted for any dosed dogs. In addition, the dosing schedule did not produce discernible venulitis in the peripheral leg veins of dogs into which it was injected.

2.2 Task Order SR01-1: Dose-Range Finding Study of Injectable Artelinate and Artesunate in Rats

2.2.1 Background and Objectives. As described above, efforts have been initiated towards the development of an iv formulation for the treatment of *Plasmodium falciparum* malaria. AL is the only member of the artemisinin class of compounds that is deemed to be both soluble and stable in water. A task order was recently initiated to investigate the pharmacodynamic effects of an AL/lysine formulation and an aqueous formulation of AS in rats. The objective of the task order is to conduct a dose-range finding evaluation of the pharmacodynamic effects of AL and AS, as assessed by signs of clinical and pathological toxicity, following iv administration of either compound to rats for 7 consecutive days. During phase 1 of this task order, an estimate of the LD₅₀ of iv administered AL or AS will be obtained. This information will be used to determine doses of AL or AS to be given during a subsequent 7 day range finding toxicity study (phase 2). The first phase of this study was initiated September 26, 2001. The range finding phase is scheduled to be initiated October 24-25, 2001. To date, no results are available from this study.

3.0 KEY RESEARCH ACCOMPLISHMENTS

Key research accomplishments during the past year of the contract included:

- Determined that an injectable formulation of AL/lysine was well tolerated by dogs given a single iv dose, equal to or greater than the anticipated clinical dose, daily for 7 consecutive days.
- Determined that discernible venulitis and other clinical signs of toxicity were not produced in dogs given a single iv dose of AL/lysine injected into the same vein daily for 7 consecutive days.

4.0 REPORTABLE OUTCOMES

To date, there have been no reportable outcomes for work completed during the past contract year.

5.0 CONCLUSIONS

Artelinic acid has been proposed as a likely drug for parental administration as an anti-malarial agent because of its chemical stability and aqueous solubility. The results of the study with iv artelinic acid in dogs indicated that an injectable formulation of artelinic acid/lysine was well tolerated by dogs given a single iv dose, equal to or greater than the anticipated clinical dose, for 7 consecutive days. This dosing schedule did not produce discernible venulitis in the peripheral leg vein of individual dogs into which it was injected or any other observable signs of clinical toxicity. These results provide support for further work related to the development of an iv formulation of artelinic acid for clinical administration.

6.0 REFERENCES

1. Institute of Animal Laboratory Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, D.C., 1996.